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Control of *apterous* by *vestigial* drives indirect flight muscle development in *Drosophila*

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Abstract

Drosophila thoracic muscles are comprised of both direct flight muscles (DFMs) and indirect flight muscles (IFMs). The IFMs can be further subdivided into dorsolongitudinal muscles (DLMs) and dorsoventral muscles (DVMs). The correct patterning of each category of muscles requires the coordination of specific executive regulatory programs. DFM development requires key regulatory genes such as *cut* (*ct*) and *apterous* (*ap*), whereas IFM development requires *vestigial* (*vg*). Using a new vg^{null} mutant, we report that a total absence of *vg* leads to DLM degeneration through an apoptotic process and to a total absence of DVMs in the adult. We show that *vg* and *scalloped* (*sd*), the only known VG transcriptional coactivator, are coexpressed during IFM development. Moreover, we observed an ectopic expression of *ct* and *ap*, two markers of DFM development, in developing IFMs of vg^{null} pupae. In addition, in vg^{null} adult flies, degenerating DLMs express *twist* (*twi*) ectopically. We provide evidence that *ap* ectopic expression can induce per se ectopic *twi* expression and muscle degeneration. All these data seem to indicate that, in the absence of *vg*, the IFM developmental program switches into the DFM developmental program. Moreover, we were able to rescue the muscle phenotype of vg^{null} flies by using the activity of *ap* promoter to drive VG expression. Thus, *vg* appears to be a key regulatory gene of IFM development.

Keywords: *vestigial*; *apterous*; *twist*; *cut*; Indirect flight muscles; *Drosophila*

Introduction

The correct patterning of differentiated tissues requires the coordination of executive genetic regulatory programs and requires several key regulatory genes. In *Drosophila*, the patterning of adult muscles is defined during embryogenesis by the expression level of *twist* (*twi*), a bHLH transcription factor (Baylies and Bate, 1996; Baylies et al., 1998). Once embryonic muscles become determined, *twi* expression fades but persists in some cells which are the progenitors of adult muscles. These progenitors proliferate during the three larval stages and are recruited to form adult muscles during metamorphosis. Myoblasts that will form the adult flight muscles proliferate on the presumptive notal part of the wing imaginal disc (Bate et al., 1991). As long as they remain on the wing disc, these proliferating myoblasts are named adepithelial cells. In the late third instar larva, the adepithelial cells are partitioned into two distinct populations. One population, located at the proximal region of the presumptive notum, expresses the Vestigial (VG) transcription factor and low levels of Cut (CT) transcription factor. The other population, located at the distal part of the presumptive notum, expresses high levels of *ct* but does not express *vg* (Sudarsan et al., 2001).

Flight muscles in *Drosophila* are subdivided into direct flight muscles (DFMs) and indirect flight muscles (IFMs). At the onset of metamorphosis, all larval thoracic muscle fibers histolyse, except for a subset, the larval oblique mus-

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cles (LOMs; three LOMs per hemisegment), which are used as templates for the formation of one set of IFMs, the dorsolongitudinal muscles (DLMs). The DFMs, however, are formed de novo, without contribution of the LOMs, by the fusion of myoblasts that have migrated from the distal part of the presumptive notum, where they express high levels of CT (Sudarsan et al., 2001). These myoblasts and developing DFMs express the LIM-homeodomain transcription factor *apterous* (*ap*) but do not express *vg* (Ghazi et al., 2000; Sudarsan et al., 2001).

The IFMs represent the majority of the thoracic muscles. They are constituted of six DLMs and seven dorsoventral muscles (DVMs) per hemisegment. The DLMs are formed by fusion between myoblasts that have migrated from the proximal part of the presumptive notum (former VG-expressing adepithelial cells) with the LOMs (Fernandes et al., 1991). About 8 h after puparium formation (APF), these myoblasts surround the three LOMs and fusion begins. Between 14 and 20 h APF, the three larval templates (LOMs) vacuolate and split into six DLMs (Fernandes et al., 1991). The fusion between developing DLMs and myoblasts is achieved at 30 h APF. Throughout this process, the myoblasts and the developing fibers express *vg* and *erected wing* (*ewg*) but do not express *ap* (de Simone et al., 1996; Ghazi et al., 2000; Ng et al., 1996; Roy and VijayRaghavan, 1998; Sudarsan et al., 2001). Laser ablation of one of the three LOMs shows that myoblasts which would normally fuse with the LOMs fuse together and generate normal adult fibers in the appropriate regions of the thorax. It appears, however, that in this case the number of adult DLMs is abnormal. Thus, the role of LOMs is limited to partitioning DLM-forming myoblasts and to generate a fixed number of DLMs (Farrell et al., 1996; Fernandes and Keshishian, 1996). Whereas LOMs are not necessary for muscle development, myoblasts are required for normal splitting and thus for DLM formation (Anant et al., 1998). The second class of IFMs, the DVMs, are formed de novo by the fusion of myoblasts that come from the wing and leg discs (Fernandes et al., 1991; Rivlin et al., 2000).

Ectopic expression experiments have shown that VG and CT expression levels are stabilized by a repressive feedback loop. Indeed, expression of high levels of CT in proximal myoblasts leads to VG repression and loss of DLMs. Moreover, overexpression of VG in distal myoblasts leads to CT repression and a reduction in DFM number (Sudarsan et al., 2001). These data suggest that two distinct developmental processes are responsible for flight muscle development. One leads to DFM formation, the other to IFM formation. Adepithelial cells entering the DFM developmental pathway express CT but do not express VG. After puparium formation, myoblasts and developing fibers express Apterous (Ghazi et al., 2000; Sudarsan et al., 2001). Adepithelial cells necessary for the IFM formation process express VG and a low level of CT. During puparium formation, VG is found in myoblasts and developing IFMs (Sudarsan et al., 2001).

In *apterous* (*ap*) mutant flies, degeneration has been observed in DFMs and IFMs. Ghazi et al. (2000) reported that the DFM phenotypes are due to an absence of AP in muscle fibers. They demonstrated that another process is responsible for DLM degeneration: they showed that AP is not normally expressed in developing DLMs but is expressed in DLM cuticle attachment sites and that its absence leads to misattachment and subsequently to degeneration. They showed that AP regulates *stripe* (*sr*) (Ghazi et al., 2000), which is necessary for muscle attachment to the epidermis (Costello and Wyman, 1986; Fernandes et al., 1996). In hypomorphic *vg* mutant flies, a reduced number of DLMs and an absence of DVMs have been reported (Sudarsan et al., 2001).

vg is known to be necessary for wing development (Bray, 1999; de Celis, 1999). During wing disc development, *vg* expression is under the control of *ap* and interacts genetically with *scalloped* (*sd*) (Paumard-Rigal et al., 1998; Varadarajan and VijayRaghavan, 1999). Dimerization of SD with VG is necessary to form a functional transcription factor (Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998).

In this study, we have investigated the role of *vg* in IFM formation. We have shown that *vg* and *sd* are coexpressed in adepithelial cells, in myoblasts surrounding the forming DLMs, and in elongating muscle fibers during metamorphosis. Using a new null allele of *vg*, we have shown that the absence of *vg* leads to IFM degeneration through an apoptotic process. We present strong evidence that this degeneration is the result of the commitment of IFM-forming myoblasts into a DFM-like developmental pathway. We show that myoblasts and developing fibers of the vg^{null} mutant express CT and AP, two specific markers of DFM differentiation that are not expressed during IFM development. Nevertheless, we show that the requirement of VG for IFM formation is a late event and that, in CT-expressing myoblasts, commitment is not definitive. Our data suggest that *vg* is a major component of IFM development.

Materials and methods

Fly stocks and heat shocks

The vg^{null} and UAS-*vg* strains were generated in our laboratory (Paumard-Rigal et al., 1998; Zider et al., 1998). The vg^{null} mutant was produced by excision of the P element inserted in the vg^{21} strain. This excision removed the eight *vg* exons without affecting neighboring genes. The mutant is homozygous viable but with reduced viability compared with wild-type flies. Females are sterile. *ap-LacZ* and *ap-GAL4* strains were obtained from S. Cohen's laboratory and the sd^{58}, sd^{3L}, and *sd-LacZ*^{ETX4} strains from the Bloomington *Drosophila* Stock Center. *MHC-LacZ* and *actin88F-GFP* are described in Hess et al. (1989) and

Barthmaier and Fyrberg (1995), respectively. The *twi-LacZ* strain is described in Thisse et al. (1991).

hsp70-ap (Bourgouin et al., 1992) flies received three 45-min heat shocks at 37°C, separated by 90-min intervals at 25°C. Pupae were heat shocked at various times (every 2–3 h) from 2 to 72 h APF.

Electron microscopy

DLMs from 3-day-old males (controls Canton wild type strain and vg^{null} mutants) were fixed for 1 h in a solution of 2% glutaraldehyde, 1% paraformaldehyde in 0.2 M sodium cacodylate buffer. After being washed three times in the buffer, samples were osmicated for 45 min in an aqueous osmium tetroxide (OsO_4) solution, serially dehydrated in ethanol, and embedded in epon–araldite. Ultrathin sections (70 nm) were stained with uranyl acetate and lead nitrate, and observed with a Philips 201 microscope.

Histology

Adult flies were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Next, 10-μm-thick sections were cut by using a Leica microtome. Sections were processed for toluidine blue staining.

Muscle preparation

Adult thoraces were fixed for 1 h in 4% paraformaldehyde and cut sagittally in PBS (phosphate saline buffer). Dissection of pupae was performed as previously described (Fernandes et al., 1991).

Immunochemistry:

Tissues were fixed for 1 h in 4% paraformaldehyde, washed three times in PBT (phosphate saline buffer, 0.3% Triton X 100), and incubated for 1 h in PBT-NGS (PBT, 4% normal goat serum) at 4°C. Samples were incubated overnight in a PBT-NGS antibody solution at 4°C. Samples were washed three times in PBT for 10 min. For fluorescence detection, samples were then incubated with fluorescently labeled secondary antibody for 2 h, washed three times in PBT, and mounted in Vectashield (Vector, Burlingame, CA). For peroxidase detection, horseradish peroxidase immunostaining was performed with the Vectastain ABC Kit according to the Vector protocol (Vector, Burlingame, CA). VG antibodies were a gift from S. Carroll and were used at a 1:200 dilution. TWI antibodies were a gift from S. Roth and were used at a 1:5000 dilution. Cut antibodies were purchased from the Developmental Studies Hybridoma Bank and were used at a 1:200 dilution. GFP antibodies were purchased from Roche Applied Science and were used at a 1:1000 dilution. Preparations were observed with a Leica TCS-SP confocal microscope.

X-Gal stainings were performed as described in Van de Bor et al. (1999).

RT-PCR experiments

RNAs from dissected DLMs were isolated with the Gibco BRL trizol reagent kit and treated with DNase. Reverse transcription and amplification reactions were performed as previously described in Zider et al. (1996). Primers for *apterous* cDNA amplification were: 5′-gtagcacatcaagggagcaa-3′ and 5′-agaggtagaagcgatcctgt-3′. Primers for *vg* cDNA amplification were: 5′-ttctcttccgattgagcggc-3′ and 5′-tattctgctttgcgatgtgg-3′. Primers for *rp49* were: 5′-tcctaccagcttcaagatgac-3′ and 5′-gtgtattccgaccacgttaca-3′.

Results

IFM degeneration is observed in vg^{null} flies

vestigial (*vg*) is expressed in adepithelial cells (Ng et al., 1996), and hypomorphic *vg* mutants show muscle phenotypes (Sudarsan et al., 2001). We looked for muscle degeneration in our *vg* amorphic mutant (vg^{null}). In wild type flies (Fig. 1A), six DLMs (arrowheads) and seven DVMs (arrows) per hemisegment are present. In homozygous vg^{null}/vg^{null} flies (Fig. 1B), some DLMs (arrowheads) are present but no DVMs can be seen. We observe that the level of degeneration increases in old flies. However, the site of muscle attachment to the epidermis appeared normal (Fig. 1E, asterisks). Observation of the 48-h APF mutant pupae revealed a normal DLM configuration (Fig. 1D, asterisks), indicating that splitting occurs normally and that DLMs degenerate during late pupal stage and in the adult. To better understand the effects of the absence of *vg* on the DLMs, an ultrastructural investigation was undertaken. Degenerating mutant muscle fibers of the adult were examined. Data from electron microscopy showed a complete disorganization of the mutant myofibrils (Fig. 2B; compare with wild type 2A) and characteristic apoptotic nuclei (Fig. 2C and D; arrow). We conclude that Vestigial is required for IFM development and that its absence leads to DLM degeneration through an apoptotic process.

Vestigial and Scalloped are expressed in myoblasts and developing DLMs

It has been previously shown that *vestigial* is expressed in adepithelial cells that give rise to IFMs in late third instar larvae (Ng et al., 1996). VG was also found in swarming myoblasts around developing DLMs and in the nuclei of some fiber (Sudarsan et al., 2001). In this paper, we will distinguish between myoblasts located on the wing disc, called adepithelial cells, and migrating myoblasts referred to as myoblasts. Twenty-one-hour APF pupae carrying a *MHC-LacZ* transgene were dissected and labeled with anti-

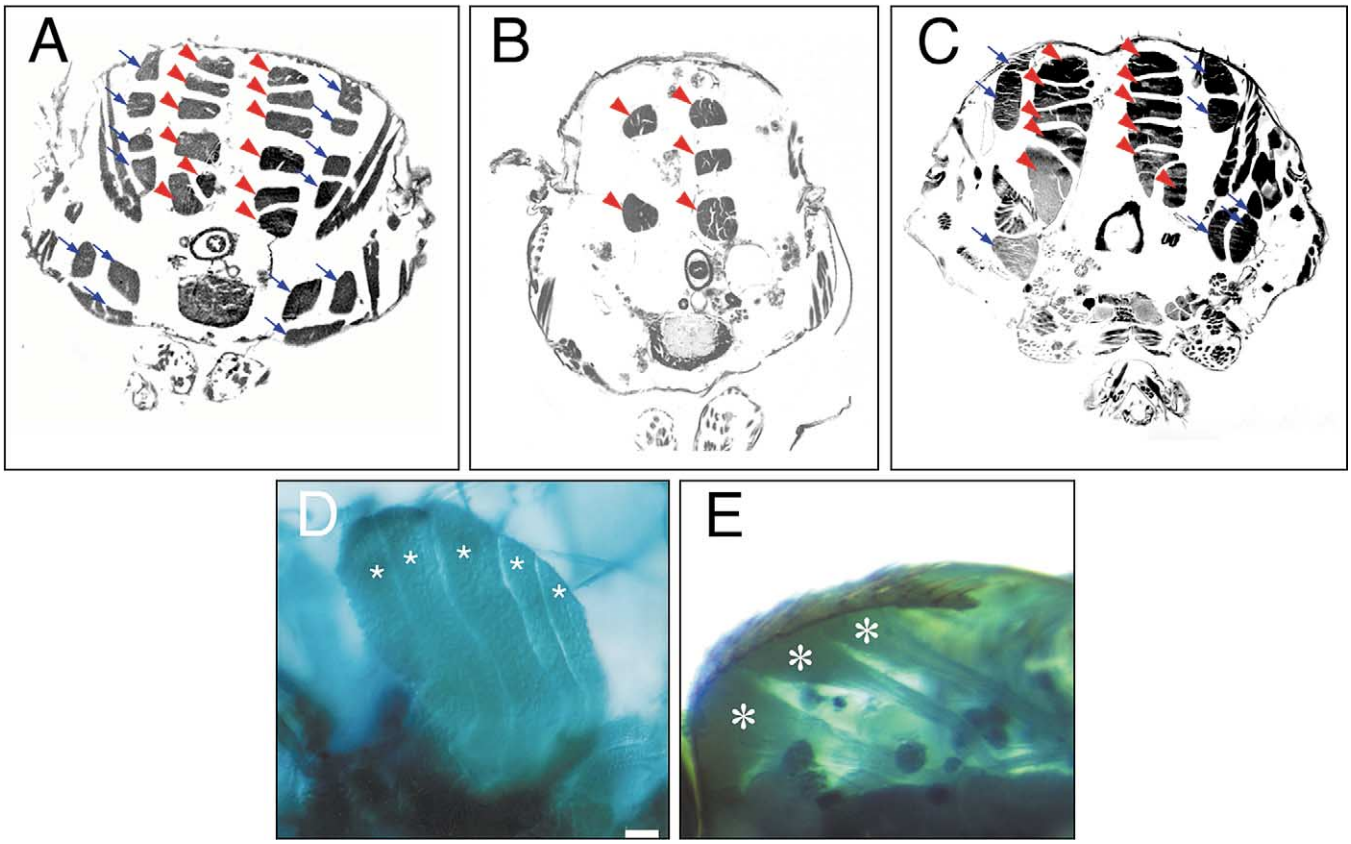


Fig. 1. Degeneration of DLMs and absence of DVMs in the vg^{null} mutant. Three transversal paraffin sections (A–C) showing a Canton wild type thorax (A) with six DLMs (red arrowheads) and seven DVMs (blue arrows); a vg^{null} mutant thorax (B) with a reduced number of DLMs (red arrowheads) and no DVMs; a thorax of vg^{null}; *ap*-Gal4; UAS-*vg* fly (C) with an almost complete rescue of the vg^{null} phenotype. We can observe five DLMs (red arrowheads) and three DVMs (blue arrows) on the left hemithorax and six DLMs (red arrowhead) and five DVMs (blue arrows) on the right hemithorax. βGAL staining of a vg^{null}; *MHC-LacZ* strain (D, E) shows a normal configuration of the DLMs in a mutant context at 48 h APF. Asterisks show developing DLMs (D). Adult thorax of a vg^{null}; *MHC-LacZ* strain cut sagittally shows no significant alteration of the attachment sites (E) (asterisks). Scale bar, 50 μm.

βGAL and anti-VG antibodies. VG is present in myoblasts swarming around the DLMs (Fig. 3A and C, asterisks) and in all developing DLM nuclei (Fig. 3A–C, arrows). It has been established that VG forms with SD a heterodimeric transcription factor (Halder et al., 1998; Paumard-Rigal et al., 1998; Vaudin et al., 1999). The interaction between these two partners is required for target gene activation during wing development. Interestingly, Halder et al. (1998) have shown that, in the absence of SD, VG is located in the cytoplasm and in the nuclei of S2 cells, while exclusive nuclear localization is observed when VG and SD are co-expressed. Therefore, we looked for *sd* expression in myoblasts and developing muscles. Using an *sd-LacZ* strain, we show that *sd* is expressed in adepithelial cells where it colocalizes with VG (Fig. 3D–F). In 21-h APF pupae, *sd* is expressed in swarming myoblasts (Fig. 3H, arrow) and in developing DLMs (Fig. 3H, asterisks). Our data thus show complete colocalization with VG (Fig. 3G, anti-VG labeling; Fig. 3I, merge). Even though we do not have any functional evidence, we can assume that, as in the wing pouch, SD and VG are partners during muscle development. However, examination of DLMs in several *sd* mutants (sd^{3L}, sd^{58}) revealed no obvious phenotype. Finally, we looked for *vg* expression in adult DLMs using RT-PCR and showed that *vg* is expressed in these muscles (Fig. 4).

cut expression is deregulated in vg^{null} context

Recent studies have shown that adepithelial cells are partitioned into two distinct populations in which VG and CT levels are stabilized by a mutually repressive feedback loop (Sudarsan et al., 2001). The distal myoblasts express a high level of VG and a low level of CT and give rise to IFMs (Fig. 5A–C, arrow). The proximal myoblasts, which do not express VG but express a high level of CT, give rise to DFMs (Fig. 5A–C, arrowhead). We wondered if the absence of VG in vg^{null} flies is associated with CT derepression. In vg^{null} mutants, no difference was observed in CT levels between proximal and distal myoblasts (Fig. 5D–F). In addition, no significant difference in adepithelial cell number was found between wild type and mutants (data not shown). Therefore, it seems that the absence of VG leads to a derepression of *cut* in all adepithelial cells. To analyze this phenomenon further, we examined CT expres-

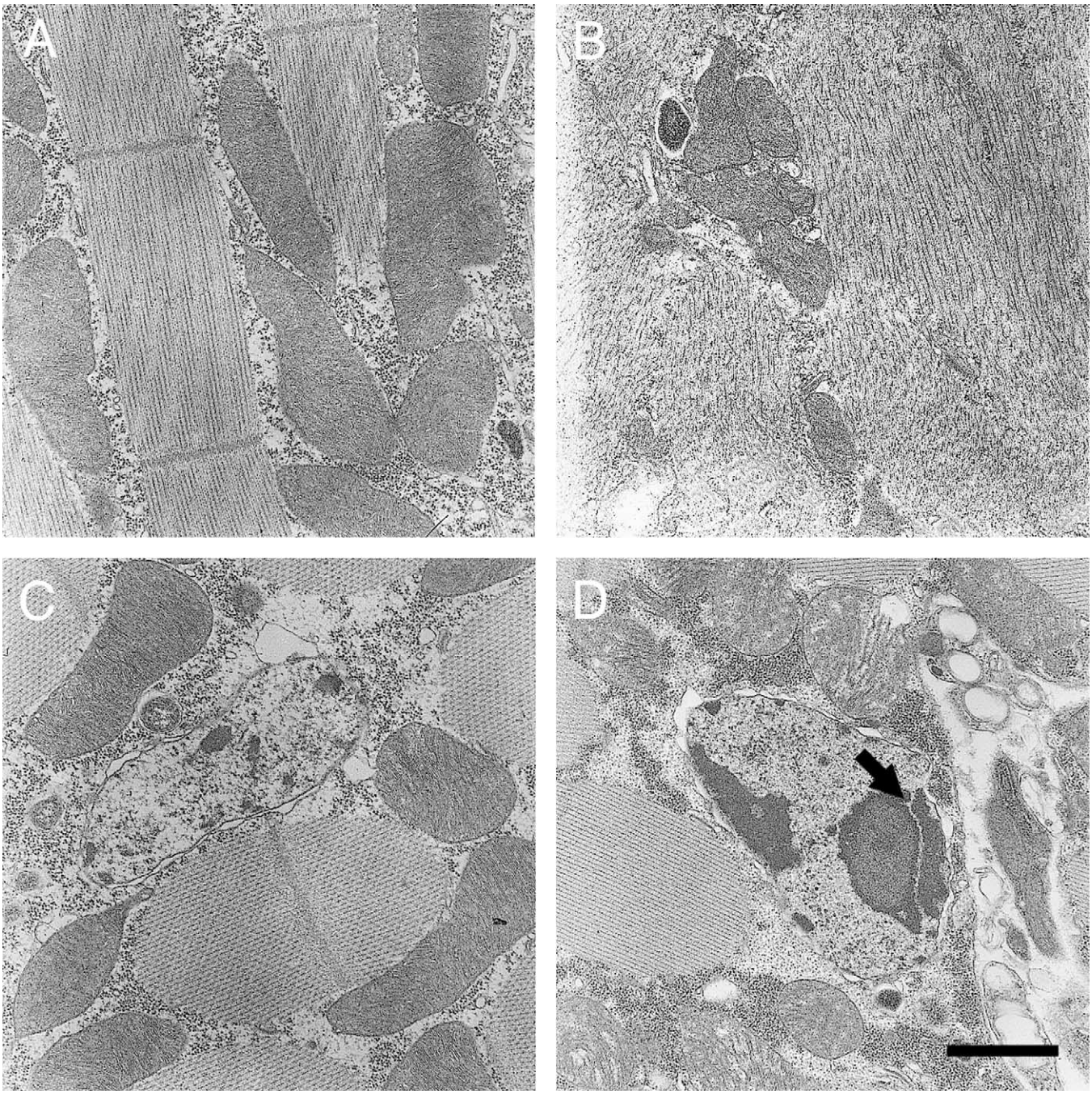
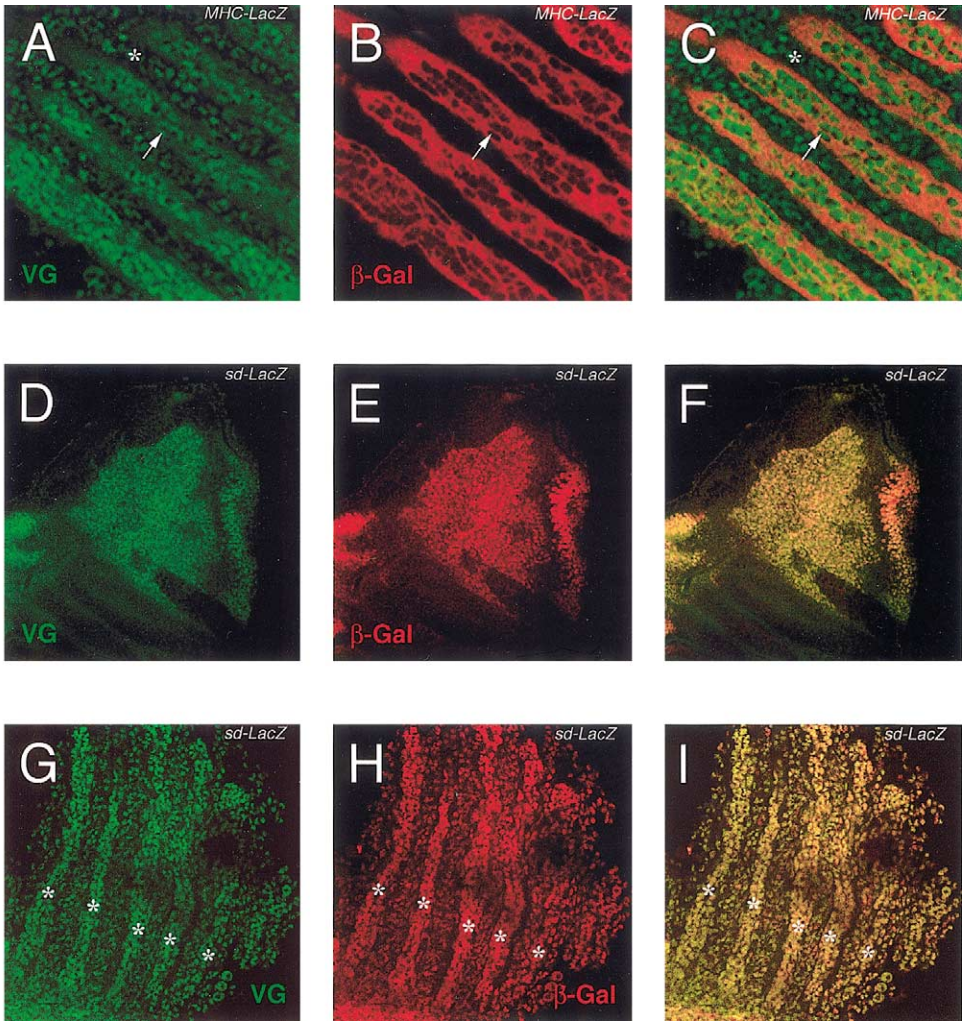


Fig. 2. Electron microscopy of adult DLMs. Canton wild type strain (A, C). vg^{null} mutant (B, D). In the vg^{null} mutant fiber, the ultrastructure is clearly abnormal compared with the control (A). Moreover apoptotic nuclei are observed in the vg^{null} mutant (D, arrow showing splitting of the nucleoli). Scale bar, 1 μm.

sion in 21-h APF pupae. No CT expression was seen in either myoblasts or developing DLMs in wild type flies (not shown). In vg^{null} mutant flies (Fig. 5G–I), CT expression was found in swarming myoblasts (arrow) and in developing muscles (asterisks). This ectopic expression of CT does not seem to be associated with a reduction in the number of myoblasts swarming around the DLMs (compare Fig. 3G with Fig. 5H) as it was shown in overexpression experiments of CT (Sudarsan et al., 2001).

apterous expression is deregulated in vg^{null} context

It has been proposed that the adepithelial cells that do not express VG while expressing high levels of CT participate in DFM development, suggesting that CT sets up a regulatory program specific to DFMs (Sudarsan et al., 2001). Since CT is ectopically expressed in vg^{null} DLM-forming myoblasts, we hypothesized that these myoblasts enter a DFM-like developmental process which leads to IFM de-


A
MHC-LacZ
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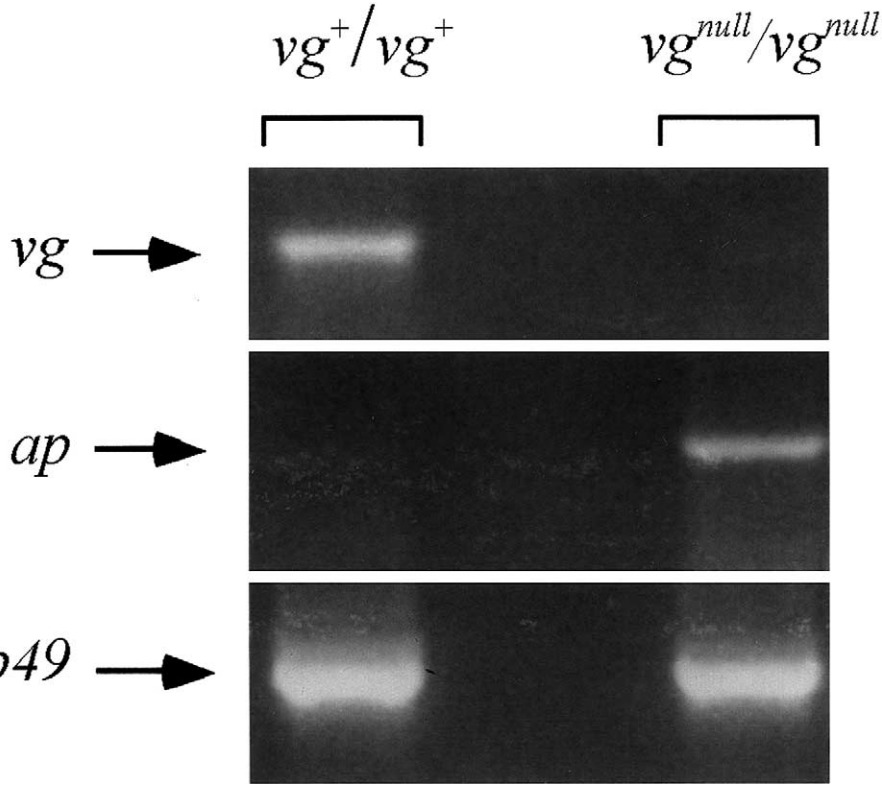
vg+/vg+
vgnull/vgnull
vg
ap
rp49

4

generation. To verify this hypothesis, we looked for the expression of a late DFM marker, *ap*, in vg^{null} developing DLMs. *ap* encodes a LIM domain protein (Bourgouin et al., 1992). Its expression has been reported in myoblasts forming the DFMs as well as in developing and adult DFMs. However, adepithelial cells do not express *ap*: *ap* expression begins 17–19 h APF in DFM-forming myoblasts (Ghazi et al., 2000). In contrast, *ap* is not expressed during DLM formation (Ghazi et al., 2000; Sudarsan et al., 2001). We looked for *ap* expression in vg^{null} mutant DLMs using an *ap-LacZ* enhancer trap. In *vg* mutant 21-h APF pupae, *ap* is ectopically expressed in myoblasts and in developing DLMs (Fig. 6A–C, asterisks). Moreover, this *ap* ectopic expression in vg^{null} developing IFMs is associated with absence of *actin 88F* expression, an IFM-specific marker (Fig. 6G and H). However, no *ap* ectopic expression was seen in adepithelial cells of vg^{null} mutants (Fig. 6D–F). We next investigated *ap* expression in adult flies. *ap* expression, detected on cryostat sections, was found in the degenerating DLMs of vg^{null} adult flies (Fig. 7B, arrowheads), but not in the wild type DLMs (Fig. 7A). *ap* deregulation was confirmed by RT-PCR experiments on DLMs. *ap* transcripts were only detected in vg^{null} DLMs (Fig. 4). These observations are consistent with our hypothesis that myoblasts are misprogrammed in vg^{null} mutants: as for DFMs, *ap* expression in DLM-forming myoblasts and DLMs begins after the onset of metamorphosis and persists in adults.

Since the *ap* promotor is activated in myoblasts and developing DLMs in vg^{null} mutants, we tested whether the muscle phenotype could be rescued by expressing VG according to the *ap* expression pattern. *ap-GAL4; UAS-vg* vg^{null}/vg^{null} flies showed a partial but significant rescue of the muscle phenotype (Fig. 1C, compared with Fig. 1B). Thus, VG expression in myoblasts and developing DLMs of vg^{null} flies during metamorphosis is sufficient to rescue the muscle phenotype, even if CT is misexpressed at earlier stages. Adepithelial cell commitment to form IFMs or DFMs is thus not definitive. Definitive commitment must be mediated by a factor other than CT. This factor could be AP.

Derepression of apterous is sufficient to induce muscle degeneration

If AP is the key factor in myoblast commitment to form DFMs rather than IFMs, its ectopic expression in myoblasts and developing DLMs should induce DLM degeneration. We expressed *ap* at various stages of development using an inducible *hsp70-ap* transgene. Muscle degeneration was observed; the most deleterious effect on DLMs was observed when the heat shocks were performed between 23 and 28 h APF (Fig. 7C). Thus, *ap* expression in developing DLMs is sufficient to induce DLM degeneration, independently of early *ct* expression.

Twist deregulation is associated with DLM degeneration in vg^{null} mutants

TWI is expressed in all adepithelial cells and swarming myoblasts around developing DLMs. Its expression shuts-off when differentiation begins (Fernandes et al., 1991). Previous studies have shown that TWI expression in myoblasts depends on the Notch (N) pathway (Anant et al., 1998). Moreover, ectopic expression of TWI in DLMs, directly or through an ectopic activation of N, leads to muscle degeneration (Anant et al., 1998). In addition, AP has been shown to be an activator of the N pathway at the wing margin (Bachmann and Knust, 1998; Irvine and Wieschaus, 1994). Since *ap* is expressed ectopically in degenerating DLMs of vg^{null} flies, we asked if *twi* is also activated in this context. As previously reported, no expression was found in wild type DLMs (Fig. 7D, *twi-LacZ*; Fig. 7G, anti-TWI immunostaining). In contrast, TWI expression was found in vg^{null} mutant DLMs (Fig. 7E, *twi-LacZ*; Fig. 7H, anti-TWI immunostaining). In order to verify that the ectopic TWI expression in degenerating fibers is mediated by *ap* derepression, heat shocks were performed in a *hsp70-ap; twi-LacZ* strain. When induction of *ap* occurs between 16 and 20 h APF, a strong expression of *twi* was observed in adults (Fig. 7F). We conclude that ectopic expression of *ap* can induce *twi* expression in DLMs. This induction is possibly responsible for degeneration of the DLMs in vg^{null} flies. TWI expression was investigated in developing DLMs of vg^{null} 21-h APF pupae: data indicate that TWI was not expressed at this stage (Figs. 5G and 6A, asterisks). Thus, ectopic TWI expression in muscles in vg^{null} mutants is a late event corresponding to a late reactivation of the gene.

Discussion

We know little about adult muscle diversification and what makes muscle identity. The adult flight musculature of *Drosophila* provides a good model to address this question. In our work, we have studied the function of *vg* during adult myogenesis in *Drosophila melanogaster*. Using a new vg^{null}

Fig. 3. VG and SD are expressed in myoblasts and developing DLMs. Staining of a 21-h APF pupa with VG (A) and βGAL (B) antibodies in a *MHC-LacZ* strain (A–C) shows the expression of VG in the myoblasts surrounding the DLMs (asterisk) and in muscles (arrow). Merge in (C) demonstrates that VG is expressed in the nuclei of the elongating muscle fibers. Imaginal wing disc of a $sd\text{-}LacZ^{ETX4}$ strain (D–F) stained with VG (D) and βGAL (E) antibodies shows perfect colocalization (merge in F) of the two proteins in the adepithelial cells. In a 21-h APF $sd\text{-}LacZ^{EXT4}$ pupa, staining with VG (G) and βGAL (H) antibodies shows colocalization of *vg* and *sd* expression (merge in I). Asterisks (G–D) indicate muscles.

Fig. 4. Expression of *ap* and *vg* in DLMs of adult wild type or vg^{null} flies. *vg* is expressed in wild type DLMs, whereas *ap* is not. In vg^{null} degenerating DLMs, no *vg* is detected and *ap* is expressed ectopically. *rp49* was used as control.

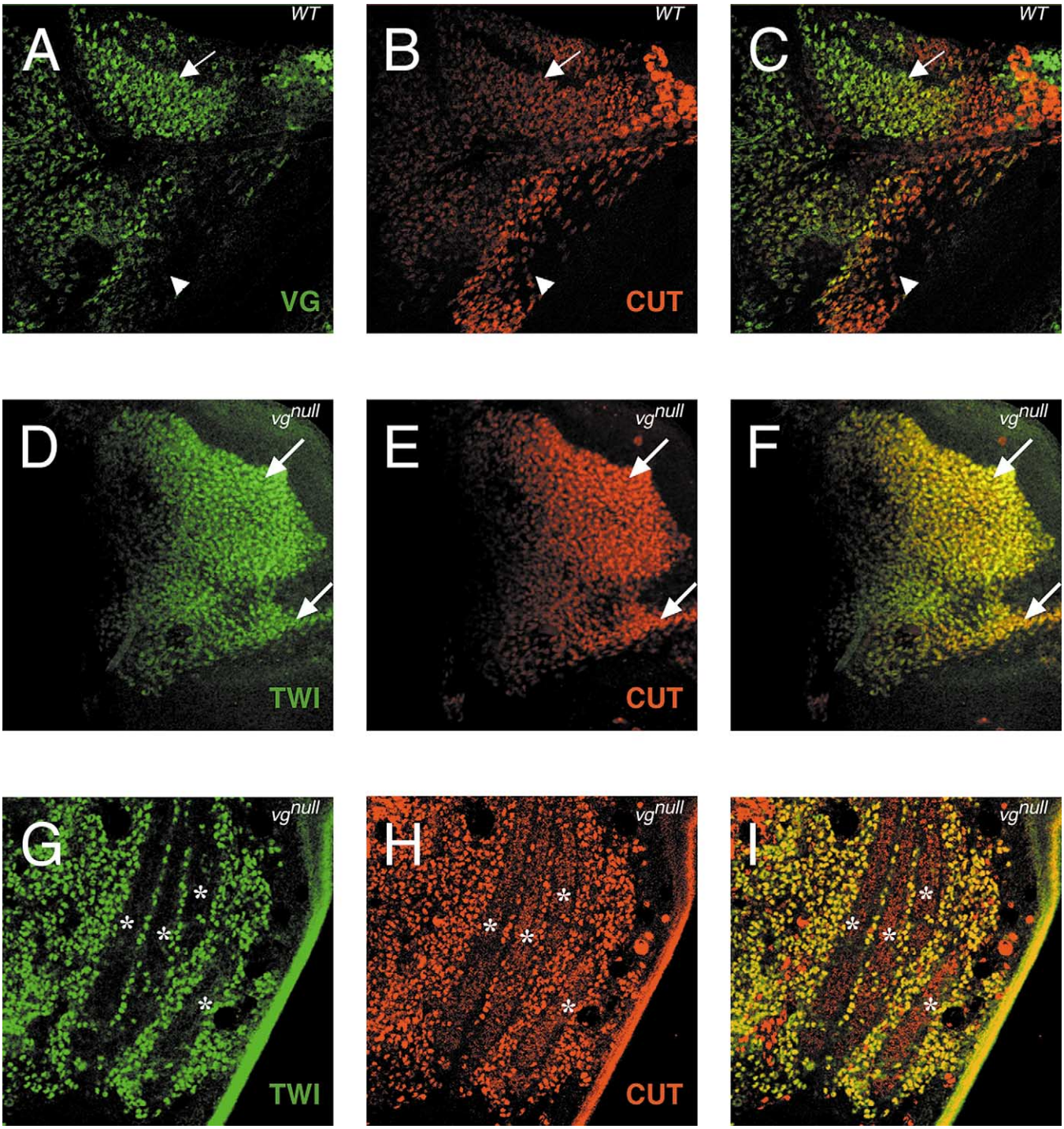

Fig. 5. CT expression is deregulated in the vg^{null} context. On imaginal wing discs of the Canton wild type strain (A–C) stained with VG (A) and CT (B) antibodies (merge in C), two populations of myoblasts are distinguishable: One does not express VG but expresses a high level of CT (arrowhead). The other expresses VG and a low level of CT (arrow). On imaginal wing discs of the vg^{null} strain (D–F), the level of CT expression is constant in all these cells (E). Anti-TWI labels all adepithelial cells (D). Merge in (F). Pupae 21 h APF of the vg^{null} strain (G–I) stained with TWI (G) and CT (H) antibodies (merge in I) show an ectopic expression of CT in swarming myoblasts and in elongating muscle fibers. Asterisks indicate the position of muscle fibers.

mutant allele, we have demonstrated that the absence of VG leads to IFM degeneration (Fig. 1A–C), while the DFMs develop normally (not shown). Some IFM phenotypes have been previously reported for the vg^{83b27R} allele, a strong allele of *vg*. In these flies, the DVMs are absent and some DLMs are missing (Sudarsan et al., 2001). Here, we show that this phenotype is fully penetrant in vg^{null} flies and that apoptosis is involved in loss of IFMs (Fig. 2). Since muscle attachment sites are normal in vg^{null} flies, the process of degeneration is different from that described in *ap* mutants

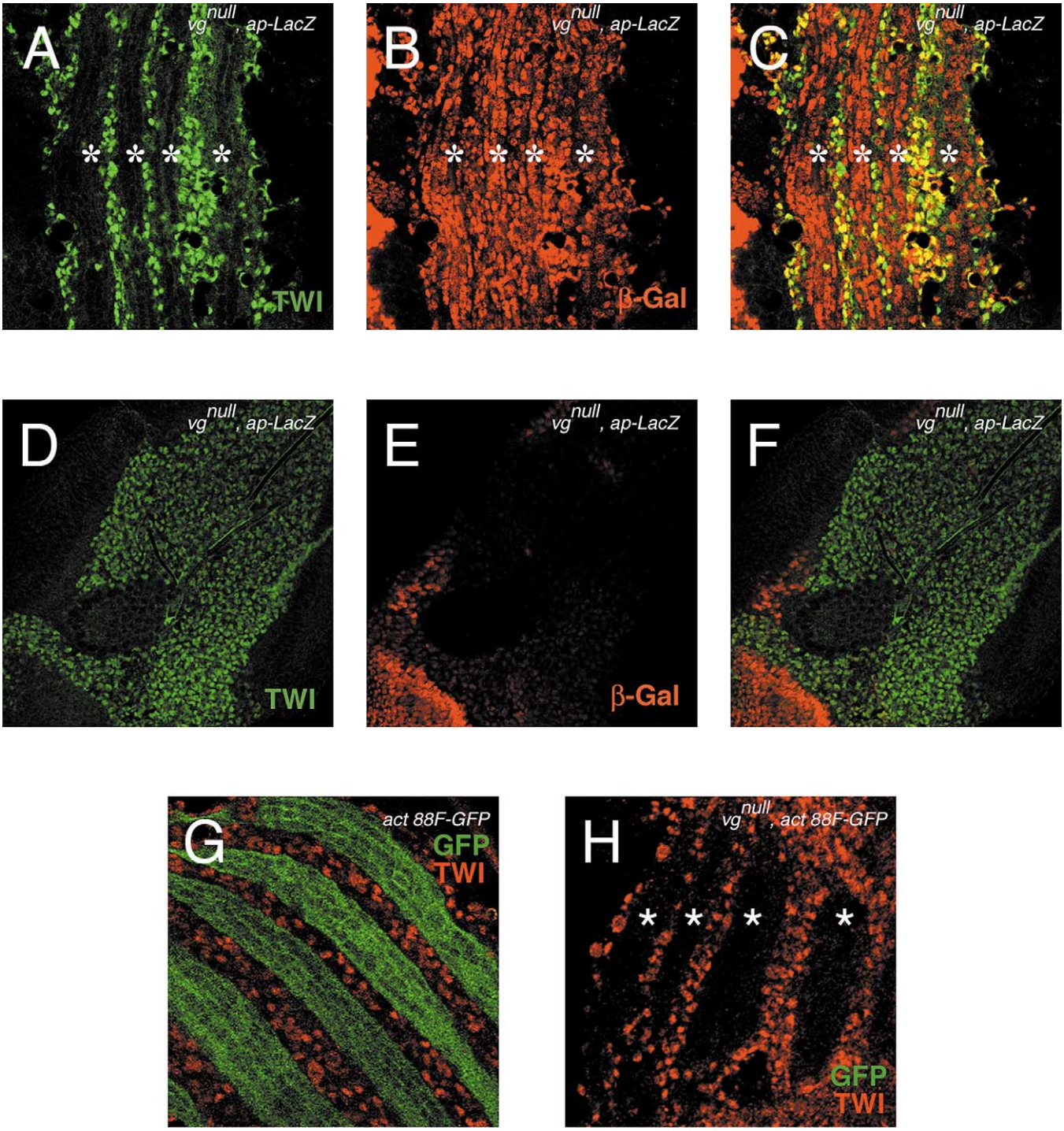

Fig. 6. *ap* expression is deregulated and *actin 88F* expression is abolished in a vg^{null} context. In pupae 21 h APF from a vg^{null}; *ap-LacZ* strain (A–C) stained with TWI (A) and βGAL (B) antibodies, we observe an ectopic expression of *ap* in swarming myoblasts and elongating muscle fibers (merge in C). Imaginal wing discs of vg^{null}; *ap-LacZ* strain (D–F) stained with TWI (D) and βGAL (E) antibodies (merge in F) show no expression of the *ap* reporter gene in the proliferating myoblasts. In 21 h pupae of vg^{+}; *actin 88F-GFP* strain (G) stained with TWI (red) and GFP (green) antibodies, we observed *actin88F* expression in developing IFMs. This expression is lost in vg^{null} context (H, asterisks). Asterisks indicate the position of the muscle fibers.

(Ghazi et al., 2000). Our phenotypic analysis finally shows that degeneration occurs during late metamorphosis (after 48 h APF).

VG interacts with SD to form a transcription factor that binds DNA through the SD TEA/ATTS domain and activates transcription through the VG activation domain (Halder et al., 1998; Simmonds et al., 1998; Vaudin et al., 1999). Since vg^{null} mutants show drastic muscle degeneration phenotypes, we decided to analyze VG and *sd* expression during development. VG expression in adepithelial

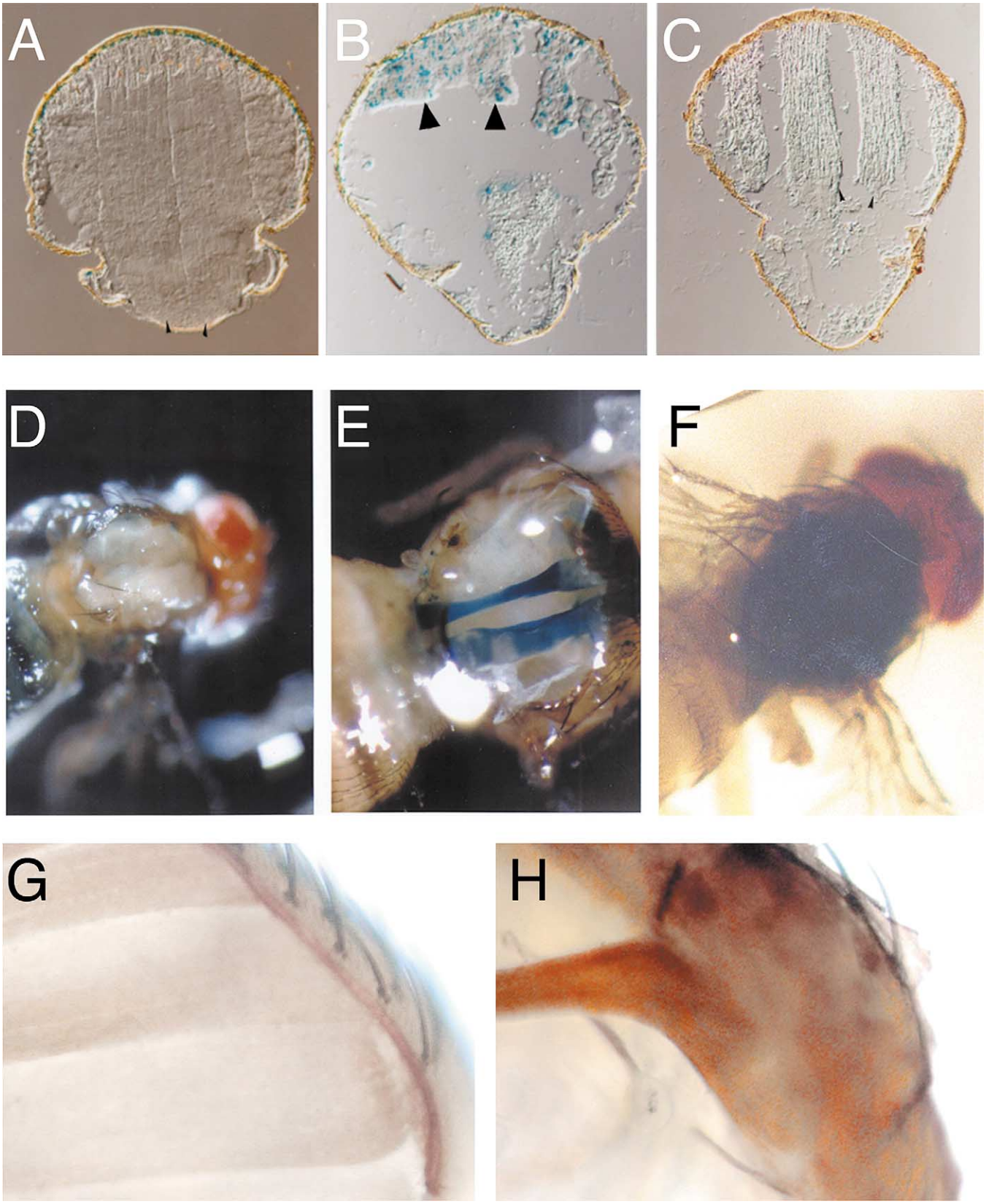

Fig. 7. Deregulation of *ap* induces degeneration of DLMs and ectopic expression of TWI. Three horizontal cryosections of *Drosophila* thorax (10 μm; dorsal plane; A–C). All thoraces are from 1- to 2-day-old flies (A–C). In an *ap-LacZ* strain, *ap* expression is not observed in DLMs (A). A vg^{null}, *ap-LacZ* mutant thorax shows ectopic expression of the *ap* reporter gene in degenerating muscle (B, arrowheads). When ectopic expression of *ap* is induced in 24 h APF (see Materials and methods), degenerating fibers are observed (C). An adult thorax of the *twi-LacZ* wild type strain (D) does not show any expression of *twi*. Flies from the vg^{null}; *twi-LacZ* strain show obvious βGAL staining in the degenerating muscle fibers (E). When *ap* ectopic expression is induced in the *hsp-ap; twi-LacZ* strain, the *twi-LacZ* reporter gene is activated (F). An adult thorax of the Canton wild type strain cut sagittally shows no expression of TWI using peroxydase immunostaining with an anti-TWI antibody (G). In contrast, in a vg^{null} context (H), strong TWI staining is observed in degenerating fibers.

cells had been previously described (Ng et al., 1996). Recent studies reported VG expression in myoblasts around the forming DLMs and in some of the DLM nuclei (Sudarsan et al., 2001). Moreover, *sd* expression has been previously described in adepithelial cells and developing IFMs (Shyamala and Chopra., 1999). Here, we show that VG is present in all DLM nuclei and that *sd* is coexpressed with VG. It is therefore likely that in muscle, as in the wing disc, SD and VG are obligate partners. This result is supported by indirect arguments: (1) VG dimerization with SD is necessary for VG activity (Halder et al., 1998; Simmonds et al., 1998). Protein interaction has been recently shown between VG and Strawberry Notch (SNO), but the function of this new partner remains unknown (Nagel et al., 2001); (2) we showed that VG is localized to the nucleus in muscles and nuclear relocalization of VG in S2 cells was proven to require the presence of SD (Halder et al., 1998). However, we did not find any muscle phenotypes in *sd* strong hypomorphic viable mutants (sd^{58} and sd^{3L}). We conclude that if SD is required for muscle development, a very low level of *sd* product is sufficient to fulfill its function. There is some precedent for this type of situation: for example, whereas CT is necessary for DFMs development, viable *ct* mutant alleles do not exhibit any muscle phenotypes (Sudarsan et al., 2001).

One of the aims of developmental biology is to determine how a given cell population undergoes specific developmental program. This means trying to determine when cell commitment is specified and what are the factors involved. Adepithelial cells were at first considered as a homogenous population that expresses TWI (Bate et al., 1991; Currie and Bate, 1991; Fernandes et al., 1991; Lawrence and Brower, 1982). Most recently, Sudarsan et al. (2001) showed that adepithelial cells can be considered as two distinct populations. The population, that forms DFMs, expresses a high level of CT and does not express VG. The second population forms IFMs and expresses VG and a low level of CT. In addition, the authors showed that CT and VG levels are stabilized by a repressive feedback loop: overexpression of CT in all myoblasts using the *1151-GAL4* driver (Roy and VijayRaghavan, 1998) leads to VG repression and to IFMs-specific apoptotic degeneration; overexpression of VG in all adepithelial cells using the same driver leads to CT repression and to DFM degeneration (Sudarsan et al., 2001). Our data in vg^{null} flies are consistent with their observations: absence of VG leads to CT derepression in adepithelial cells (Fig. 5E), in myoblasts surrounding DLMs and in developing DLMs (Fig. 5H). However, CT overexpression phenotypes in DLMs are slightly different from those observed in vg^{null} flies. We showed that splitting of the three larval templates (LOMs) into six DLMs occurs normally and that at 48 h APF DLMs are morphologically normal in vg^{null} flies (Fig. 1D). We conclude that DLM degeneration is a late event in vg^{null} mutants. In contrast, CT overexpression leads to early degeneration of myoblasts and muscle fibers (Sudarsan et al., 2001). This suggests that CT overexpression induces apoptosis independently of VG repression. Therefore, it would seem that the effect of CT overexpression using the *1151-GAL4* driver is not equivalent to that observed in the absence of VG.

We have shown that in vg^{null} mutants all adepithelial cells express high levels of CT, while this is normally only the case of DFM-forming myoblasts (Sudarsan et al., 2001). We therefore wondered if DLM degeneration in vg^{null} mutants is the result of engagement of DLMs toward a DFM-like differentiation process. To answer this question, we decided to look for *ap* expression in vg^{null} developing and adult DLMs. In wild-type flight muscles, *ap* expression is specific to DFMs and begins at 17–19 h APF (Ghazi et al., 2000; Sudarsan et al., 2001). In vg^{null} flies, we found *ap* expression in developing DLMs at 21 h APF, in myoblasts surrounding DLMs and in adult muscles (Figs. 6B and 7B). Moreover, we observed an absence of *actin 88F* expression in vg^{null} developing IFMs, suggesting that IFM differentiation is disrupted (Fig. 6H). Interestingly, as in wild-type flies, no expression was found in adepithelial cells (Fig. 6E). These data show that *ap* starts to be expressed at the same stage in DLMs of vg^{null} flies and in DFMs of the wild type strain

In summary, we have shown that in vg^{null} flies: (1) DLM-forming myoblasts express high levels of CT, an early marker for DFM-forming myoblasts and (2) myoblasts and developing and adult degenerating DLMs express *ap*, a specific late DFM marker, whereas *actin 88F* expression, an IFM-specific differentiation marker, is lost. According to these data, we can suppose that in the vg^{null} mutants, adepithelial cells and developing DLMs enter into a DFM-like development. The suggestion that *ap* ectopic expression may impose a DFM identity on the IFMs has been previously proposed (Ghazi et al., 2000). However, an IFM-to-DFM transformation was not observed, rather IFMs degenerated through an apoptotic process. Similarly, DFMs were not transformed into IFMs upon overexpression of VG in DFM-forming myoblasts. Instead, DFM degeneration was obtained (Sudarsan et al., 2001). This suggests that VG and AP are major actors but are not sufficient for IFM and DFM development, respectively. Other signals and factors must be required to specify these muscles. It has been previously shown that nerve–muscle interaction is associated with IFM development (Fernandes and Keshishian, 1998). Kozopas has shown that *Wnt oncogene analog 2* (*Dwnt-2*) expression is required in the vicinity of the developing DFMs for patterning of DFMs (Kozopas and Nusse, 2002). Thus, it appears that adult muscle development requires complex interactions between several kinds of signals delivered in specific localizations. In vg^{null} homozygous flies, adepithelial cells and swarming myoblasts express DFM markers, but their position on the wing imaginal disc (Fig. 5D–F) and in the pupa (Fig. 5G–I) remains unchanged with respect to wild type. Thus, developing IFMs receive IFM signaling (at least nerve–muscle interactions), but myoblasts express *apterous*, a DFM maker. Moreover, they lack information

necessary for formation of either DFMs or IFMs (absence of *vg* expression). We suggest that IFM degeneration in vg^{null} homozygous flies is the result of this complex interaction between two contradictory signals (IFM and DFM) associated with incomplete signaling for formation of either type of muscle.

We tried to rescue the vg^{null} muscle phenotype by targeted VG overexpression using the UAS-GAL4 system (Brand and Perrimon, 1993). Significant rescue was obtained with the *ap-GAL4* driver (Fig. 1C). It is therefore likely that ectopic activation of the *ap-GAL4* transgene in vg^{null} DLMs and myoblasts occurs when VG is required for DLM formation. Since *ap* activation in vg^{null} myoblasts and developing DLMs occurs after puparium formation (see Fig. 6), we conclude that a late VG expression is sufficient to restore the DLM developmental process. This implies that adepithelial cell determination by the level of CT at the wing disc is reversible. Thus, even though earlier CT levels distinguish two adepithelial cell populations that will differentiate into DFMs or IFMs, definitive DFM versus IFM determination is a later event that takes place during metamorphosis. *vg* and *ap* could be key genes during specification of IFMs and DFMs, respectively. To support this hypothesis, we showed that ubiquitous overexpression of *ap* is sufficient to induce specific DLM degeneration (Fig. 7C). The way in which AP and VG direct muscle development toward a DFM or IFM fate remains unclear. However, it is well known that muscle fibers express specific structural genes or isoforms (for review, see Bernstein et al., 1993). Since *ap* and *vg* encode transcription factors, they are probably involved in specific genes activation. For example, Ghazi et al. (2000) reported that misexpression of *ap* in developing IFMs represses the expression of *actin 88F*, an IFM-specific actin gene. Moreover, no *actin 88F* expression was found in a vg^{null} context (Fig. 6G and H). However, we do not know for the moment whether AP or VG can directly activate or repress structural genes. Interestingly, the SD mammalian homolog (Transcription Enhancer Factor-1, TEF-1) has been shown to bind muscle-specific promoters, like the cardiac *α-Myosin Heavy Chain* (Gupta et al., 1997) and the cardiac *Troponin T* (Butler and Ordahl, 1999) promoters. It is therefore possible that the SD-VG dimer plays a similar role in *Drosophila*, directly activating structural genes. Further studies are necessary to address this question.

We have shown that DLMs degenerate by apoptosis in homozygous vg^{null} flies. This degeneration could be due to a misprogramming of myoblasts surrounding DLMs during development. The process that leads to apoptosis in these muscles remains to be determined. Here, we show that DLM degeneration is associated with an ectopic expression of TWI transcription factor (Fig. 7D–H). Previous studies have shown that during flight muscles development TWI expression is restricted to myoblasts (Fernandes et al., 1991) and that persistent expression in developing muscles leads to muscle degeneration (Anant et al., 1998). Thus, TWI expression in vg^{null} mutants could be responsible for DLM degeneration. Finally, we have shown that ectopic *ap* expression induces TWI expression in DLMs (Fig. 7F). Since AP and *twi* are known to be, respectively, activator and target of the N pathway (Anant et al., 1998; Bachmann and Knust, 1998; Irvine and Wieschaus, 1994), we can hypothesize that AP activates TWI ectopically in vg^{null} DLMs through the N pathway. If this hypothesis is confirmed, we can ask why AP does not activate TWI during normal DFM development. It is likely that numerous genes, other than *vg* and *ap*, are differentially activated during DFM and IFM development. TWI activation by AP could be repressed by one of these genes during DFM development.

In this study, we provided evidence that *vg* is required to change DFM-forming myoblasts into IFM-forming myoblasts. As in wing development where VG is considered as a selector gene (Kim et al., 1996), VG could be a key gene in IFM specification. Its function would be equivalent to that of AP for DFM development. DFM fate inhibition through repression of *ct* and *ap* by VG seems therefore to be a key regulation feature of IFM development. Thus, correct programming and regulation of these three genes are necessary for correct patterning of *Drosophila* flight muscles.

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